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Construction of a Plasmid that Increases the Level of *ompA* Gene Expression in *Escherichia coli* for the Study of its Effect on Bacterial Conjugation

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Conjugation, a mechanism by which bacteria exchange their genetic materials, is of great importance due to its implication in horizontal gene transfer and adaptation. The outer membrane protein A (OmpA) present on the conjugation recipient cell has previously been shown to be essential in transfer of F plasmid. The goal of this study was to construct a plasmid that could increase the level of *ompA* expression in the conjugation recipient strains, *Escherichia coli* 2320 and χ478. This plasmid could be transformed into the recipient cells for the further investigaton of how *ompA* expression affects the conjugation efficiency. Primers were designed for the amplification of *ompA* by Polymerase Chain Reaction and for the subsequent cloning of *ompA* into pCR2.1-TOPO vector using TOPO TA Cloning system. The resulting plasmid was named pCCK06-1 and was successfully isolated and transformed into *E. coli* 2320 and χ478 through electroporation. A control conjugation between F^+ donor *E. coli* JCFLOxK1200 strain and the wild-type recipient 2320 and χ478 strains was shown to be successful. Further conjugation experiments between the donor and transformed recipients could yield results of the effect of increased *ompA* expression on conjugation efficiency.

Many bacteria from different species frequently exchange their genetic materials by a process called conjugation, which occurs by cell-to-cell contact. This process has been extensively studied because of its significance in horizontal gene transfer and the resulting spread of multiple antibiotic resistance and virulence, as well as in genetic manipulation studies (4). Identifying factors that affect conjugation efficiency can be extremely useful in terms of clinical and experimental microbiology. Various studies have shown that certain plasmids with mobilizable genetic elements and an origin of transfer (ori_T) can be transferred to another cell through a pilus formed by the gene products of the *tra* operon (11). Highly conjugative plasmids, such as the F plasmid, contain their own *tra* genes for mobilization (7). Once the F pilus is assembled in the outer membrane of the donor cell, the TraN, protein assembled at the tip of the pilus, interacts and binds to the recipient cell. This interaction allows the mating pair to stabilize, which precedes the transfer of the conjugative plasmid from the donor to the recipient cell (10). A loss of function mutation in TraN has been shown to lead to a tremendous decrease in the efficiency of liquid medium conjugation (9, 10).

It has been previously discovered that the outer membrane protein A (OmpA), found abundantly on the recipient cell outer membrane (12), is the target for F pilus binding (1). Recent studies have revealed that OmpA is essential in conjugation based on the result that no conjugation was observed in *ompA* knock-out recipients (9). There has not been any research on the effect of increased level of OmpA on conjugation. Studies that have attempted to increase the level of a gene expression within cells involved cloning of the gene cassette of interest into a vector plasmid under an inducible promoter, and transforming the vector into competent cells (8). The vector pCR2.1-TOPO contains a P_{lac} promoter, which allows a high expression of the downstream genes upon induction with IPTG. In addition, it contains several markers including *lacZα*, ampicillin resistance, and kanamycin resistance, which allow easy selection and screening for desired clones. As a part of the investigation to determine if increasing the level of OmpA in the outer membrane increases the conjugation efficiency, a plasmid with a cloned *ompA* gene was constructed for the purpose of increasing the level of *ompA* expression in conjugation recipient cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* JCFLOxK1200 (F'*lac*, *nal*, *Δlac, ΔU124, nadA, aroG, gal, attL, bio, gyrA*) was chosen to be the donor strain for conjugation experiments. *E. coli* 2320 (F^-, *str*, *lacZ*, *lacY*, *thi-1*) and χ478 (F^-, *thi-1, metE, proC, purE, trp, lysA, leuB, xyl, azi, ara, lacZ, str*) were the strains used as the recipients for conjugation. All strains were supplied from MICB 421 frozen stock, UBC. Cultures were grown in LB broth (2) at 37ºC in shaking waterbath. One microgram per millilitre thiamine was added as a supplement for the recipient cultures. One hundred microgram per millilitre streptomycin or ampicillin was added to LB to prepare LB Sm or LB Amp plates. Eighty microlitre of 20 mg/mL X-gal and 125 µL of 8 mg/mL IPTG were added to plates for blue white screening.

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Conjugation. Overnight cultures of *E. coli* JCFLOxK1200, 2320, and χ478 strains were grown, diluted, and mixed for conjugation as previously described (3). One hundred microlitre of the diluted donor culture, recipient culture, and conjugation mixture were plated onto LB X-gal IPTG, LB thi X-gal IPTG, and LB Sm thi X-gal IPTG respectively, and incubated at 37ºC overnight.

Primer design for *ompA* gene. *OmpA* gene sequence was taken from *E. coli* K-12 MG1655 (1018236…1019276; NCBI GenBank GI: 48994873). Using PrimerQuest (2002; IDT Technology) and Primer Designer (v. 2.01; Scientific and Educational Software) software. OmpA-F primer (5'-GGATGATAAC**GAGG**CGCAAA-3') and OmpA-R primer (5'-GGCATTGCTGGCTAAGGAAT-3') were designed. The bolded region in the OmpA-F primer indicates the Shine-Dalgarno region for *ompA* gene (13). The primers were blasted against the *E. coli* K-12 genome to test for non-specific binding. Primers were ordered from IDT Technology (Coralville, IA).

Chromosomal DNA isolation. DNA isolation and purification were performed using the Hancock Laboratory Method (6). *E. coli* 2320 was grown overnight in 50mL LB and the chromosomal DNA was extracted and purified with 1:1 phenol:chloroform, followed by precipitation with isopropanol and 0.1 M ammonium acetate. Subsequently, ammonium acetate was washed away by 70% ethanol, and the purified chromosomal DNA was resuspended in TE.

PCR amplification of *ompA* gene. A master mix enough for five reactions was prepared in the laminar flow hood using 181.25 µL sterile water, 25.0 µL 10x PCR buffer (Invitrogen; Catalog No. 1200007), 10.0 µL 50 mM $MgCl_2$ (Invitrogen; Catalog No. 1228940), 6.25 µL 10 mM dNTPs (Invitrogen; Catalog No. 18427-013), 10 µL 25 pmol each of OmpA-F and OmpA-R primers, and 1.25 µL 5 U/µl Taq polymerase (Invitrogen; Catalog No. 1179680). The master mix was divided into five equal portions of 48 µL volumes. Two microlitre of the isolated chromosomal DNA was added into the cap of each tube, spun down briefly, and placed into Biometra T Gradient thermal cycler machine set to run for 40 cycles of denaturation at 94°C for 30 seconds, annealing at a temperature gradient of 60ºC, 60.9 ºC, 63.2 ºC, 66.8 ºC, and 69.8 ºC for 1 minute, and extension at 72ºC for 1.5 minutes. The final extension was left at 72 ºC for 10 minutes, and kept at 10 ºC until collected the next day.

Agarose gel electrophoresis. Agarose gels (2% for restriction digested PCR products, and 0.7% for restriction digested plasmids) were used to run PCR products and restriction digested plasmids, respectively. The gels were run at 100 V for approximately 1.5 hours. After electrophoresis, the gels were stained in 0.2 µg/mL ethidium bromide bath for 20 minutes. Photographs of the gels were taken by AlphaImager software (v. 4.1.0; Alpha Innotech Corporation). Gels whose band intensities had interference by high concentrations of 6x DNA buffer were submerged in distilled water overnight to allow diffusion out of 6x DNA buffer.

TOPO TA cloning of *ompA* into pCR®2.1-TOPO® vector. The o*mpA* PCR product was cloned into the vector pCR2.1 by TOPO TA Cloning (Invitrogen; Catalog No. K4500-01) to generate plasmid pCCK06-1.

Extraction of plasmid DNA in the cloning mixture. Thirty microlitre of 1:1 phenol:chloroform was added to the ligation reaction from TOPO TA cloning, and centrifuged at 13,000x g for five minutes. The top layer was removed into a new microfuge tube followed by the addition of 100 µl salted ethanol (95%). The mixture was then incubated at -20ºC for 30 minutes and centrifuge at 4 ºC for 15 minutes at 13,000x g. The supernatant was discarded and the pellet was resuspended in 100 µl of 80% ethanol. Centrifugation at 4 ºC for 15 minutes at 13,000x g was repeated and the pellet was again resuspended in 100 µL 80% ethanol. The ethanol was dried on a vacuum dryer (Eppendorf Vacufuge™, Brinkmann Instruments Inc., Westbury, NY) and the plasmid DNA was suspended in 10 µl TE buffer.

Transformation of pCCK06-1 into competent cells. pCCK06-1 was transformed into competent TOP10 cells (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G, provided by Invitrogen TOPO TA Cloning kit) by electroporation (MicroPulser™, BIO-RAD, Hercules, CA). The transformation mixture was plated on a LB Amp X-gal IPTG plates for selection of transformants and for blue-white screening for presence of the cloned insert in the plasmid. The same procedure was done to transform electrocompetent *E. coli* 2320 and χ478 strains (see below), and the resulting solutions were plated in LB Sm Amp thi plates to select for the transformants.

Plasmid isolation. Plasmids were isolated from the white TOP10 transformant colonies by using the PureLink HQ Mini Plasmid Purification Kit (Invitrogen; Catalog No. K2100-01). DNA quantity and purity was assessed by measuring absorbance at 260.0 and 280.0 nm wavelengths.

Restriction digest. The isolated plasmids from white TOP10 transformant colonies were digested with restriction enzyme *Eco*RI. Restriction digest mixture contained 10 µl of plasmid sample, 2 µl of 10x React 2 buffer, 8 µl of dH_2O, and 1 µl of *Eco*RI. The mixture was incubated for 1 hour at 37 ºC. The PCR product of *ompA* amplification was also digested with restriction enzyme *Hae*II, using the same mixture except using React 3 buffer instead of React 2 buffer, and *Hae*II instead of *Eco*RI.

Generating competent recipient cells. In order to transform *E. coli* 2320 and χ478 by electroporation, the cells were made electrocompetent (MicroPulser™ Electroporation Apparatus Operating Instructions and Applications Guide, BIO-RAD, Hercules, CA). The electrocompetent cells were transformed with the isolated pCCK06-1 from the white TOP10 transformant colonies as described above.

RESULTS

Conjugation. The colonies on the donor cell plate were all blue. Both recipient cell plates had mixtures of white and blue colonies. These blue colonies were shown to be streptomycin sensitive contaminants since they were unable to grow on LB Sm plates. The conjugation mixture plate showed two blue colonies and 100 white colonies for the conjugation with 2320.

PCR amplification of *ompA* gene. PCR amplification of *ompA* gene results are shown in Fig. 1. Two bands of similar molecular weight of the desired 1159 bp *ompA* PCR product were produced at lower annealing temperatures (60ºC, 60.9 ºC, 63.2 ºC, and 66.8 ºC). The intensity of the lower molecular weight band decreased as the annealing temperature increased. There was not much difference in the intensity of the higher molecular weight band over the five different annealing temperatures. To confirm the PCR product was in fact the desired *ompA* gene, the sample run at annealing temperature of 63.2 ºC (lane 5) was digested with *Hae*II. The results show that there are three major bands at approximately 655 bp, 314 bp, and 190 bp (Fig. 1, lane 2). The PCR sample amplified at the highest annealing temperature (69.8 ºC) does not have any non-specific products.

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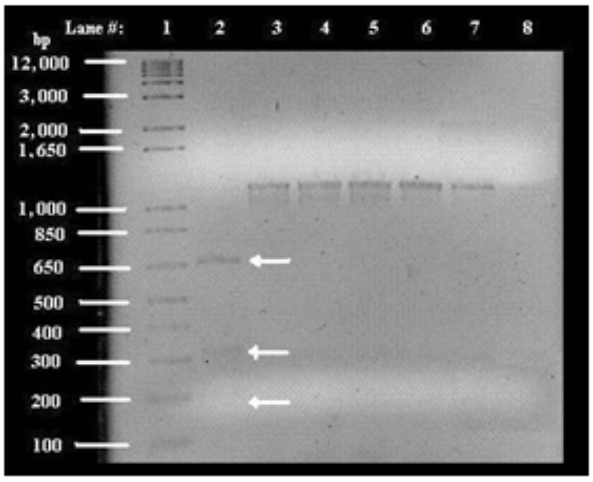

FIG. 1. PCR amplification of *ompA*. Lane 1, 1 kb plus DNA molecular weight standards (Invitrogen; Catalog No. 1273450). Lane 2, *Hae*II RE digest of the PCR product from tube #3 (lane 5). Expected products of 190 bp, 314 bp and 655 bp are indicated by the arrows. Lanes 3-7, PCR products from tubes #1-#5, in that order. The annealing temperatures for the five PCR tubes were as follows: tube #1: 60°C, tube #2: 60.9 °C, tube #3: 63.2 °C, tube #4: 66.8 °C, and tube #5: 69.8 °C. Lane 8, PCR negative control.

The restriction digested products of PCR sample in lane 7 is shown in Fig. 2. Two bands (655 bp and 314 bp) are clearly visible, while the 190 bp band is very faint near the bottom of the gel (Fig. 2, Lane 3). Figure 2 confirms that PCR tube #5 (Fig. 1, lane 7) contained a pure amplification of the desired o*mpA* gene, and this sample could be used in the subsequent cloning procedures.

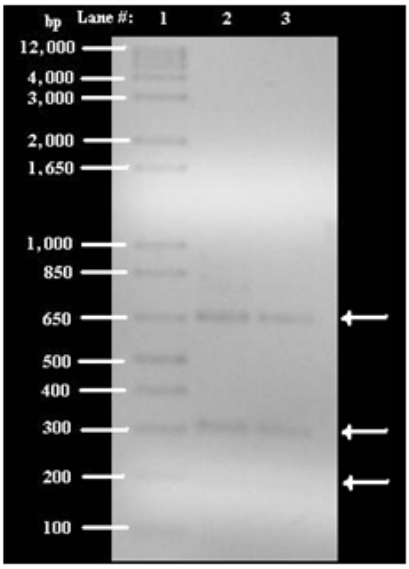

FIG. 2. Restriction enzyme digestion of PCR products. Lane 1, 1 kb plus DNA molecular weight standards (Invitrogen; Catalog No. 1273450). Lanes 2-3, *Hae*II digestion of PCR products from tubes #3 and #5 (Fig. 1), respectively. Expected fragments of 190 bp, 314 bp and 655 bp are indicated by the arrows. Extra faint bands can be seen in lane 2 while lane 3 only contains 3 bands.

TOPO cloning of *ompA* and transformation by electroporation. Transformation colonies were too numerous to count but the ratio of the number of white colonies to blue colonies was approximately 1:1. Plasmid isolation was performed on four of the white colonies present on this TOP10 transformant plate. Isolated plasmids from 4 white colonies demonstrated 2 bands (~3.9, ~1.2 kb) when digested with *Eco*RI (Fig. 3). When these plasmids were introduced into the 2320 and χ478 recipient cells, the resulting plates showed 40 colonies for 2320 transformants and 39 colonies for χ478 transformants. Two of these colonies from each of the plates were then subjected to plasmid isolation, restriction digestion with *Eco*RI, and agarose gel electrophoresis to confirm the presence of *ompA* gene insert in their plasmids (Fig. 4). Two bands are present in all of the samples: one band at ~3.9kb, and the other band at ~1.2 kb.

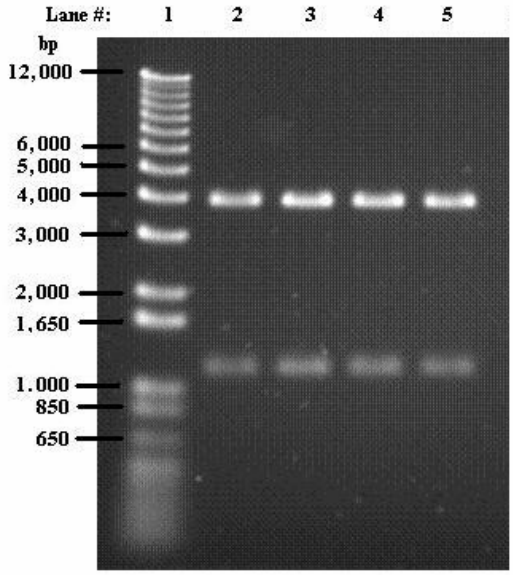

FIG. 3. Isolated and digested plasmids from TOP10 transformants. Lane 1, 1 kb plus DNA molecular weight standards (Invitrogen; Catalog No. 1273450). Lanes 2-5, plasmids isolated from four different white TOP10 transformants colonies.

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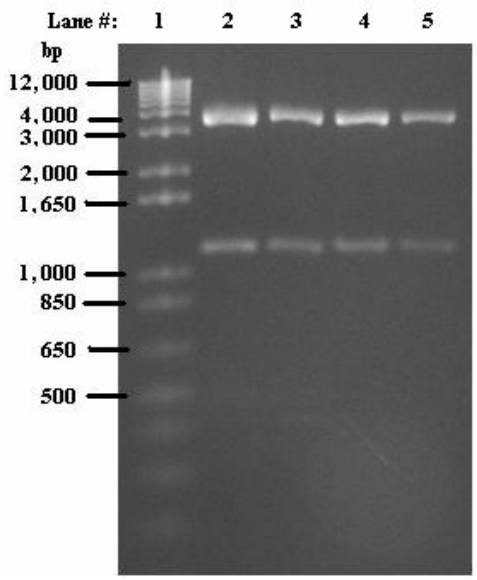

FIG. 4. Isolated and digested plasmid from recipient transformants. Lane 1, 1 kb plus DNA molecular weight standards (Invitrogen; Catalog No. 1273450). Lane 2-3, plasmid isolated from two different *E. coli* 2320 transformant colonies. Lane 4-5 plasmid isolated from two different *E. coli* χ478 transformant colonies.

DISCUSSION

E. coli strain JCFLOxK1200 has previously been shown to be a suitable conjugative donor (3). *E. coli* 2320 and χ489 were chosen to be candidates as recipient strains because of their genotype of F^-, str^R, and $lacZ^-$. Theoretically, the conjugative plasmid F'*lac* from the donor strain can be transferred to the recipient strain to confer $lacZ^+$ genotype in the recipients, which can be used to screen for transconjugants. Streptomycin resistance gene is a useful marker for selecting for the recipients against the donor. In order to confirm the suitability of 2320 and χ489 strains as conjugation recipients, control experiments were carried out as described above. Since the donor cells have F' plasmids that encode a *lacZ* gene complementing the chromosomal *lacZ* deletion, they are expected to produce β-galactosidase that will cleave X-gal and give rise to blue colonies. The two recipient strains are expected to give rise to white colonies on X-gal IPTG media because of the $lacZ^-$ genotype. The recipient cells that received the F plasmid during conjugation (transconjugants) would also turn blue similar to the donor because of the *lacZ* present in F. This could be used to distinguish between transconjugants and non-transconjugants among recipient cells by blue-white screening. The results showed two blue colonies and 100 white colonies on the LB Sm thi X-gal IPTG media that had the conjugation mixture for 2320 plated. This meant that the two blue colonies are streptomycin resistant and therefore are 2320 strains and not the contaminants. These are also transconjugants because they are $lacZ^+$ most likely due to acquisition of F'*lac*. The corresponding plate for χ478 had only one white colony, which did not provide any useful result for its suitability as conjugation recipient. These results demonstrated that the strain 2320 would be a suitable candidate for conjugation recipient, with 2% conjugation efficiency. A positive control for conjugation under these experimental conditions was thus established.

In order to ensure that the entire *ompA* gene cassette is cloned, the primers had to flank upstream and downstream of the start and stop codons of the gene. Membrane proteins usually have an N-terminal signal sequence that has an important function for targeting the protein for export or for insertion into membrane (14). Since OmpA is an outer membrane protein, it is most likely that its N-terminal amino acid sequence will serve the same function. This implies that if the N-terminal region is not included by the primers and thus is not amplified, the partial *ompA* gene might not be expressed successfully or be incorporated properly into the outer membrane. In addition, if the coding region of *ompA* gene was inserted into a plasmid somewhere downstream of an inducible promoter, a miscellaneous portion of the plasmid upstream of the cloned gene would be translated together and might interfere with the N-terminal signalling. To avoid this possibility, the primers were designed also to include a ribosome-binding site (Shine-Dalgarno sequence) in its proper position relative to the *ompA* gene. This would allow transcription of the gene to start from the inducible promoter while translation would be done independently starting from the SD region to produce a native OmpA protein.

The expected PCR product is 1159 bp long, containing the entire *ompA* gene which is 1041 bp in length. The putative primer sequences were blasted against the whole genome of *E. coli* K-12 chromosome to make sure that the primers would not bind to another part of the chromosome other than at the *ompA* gene and thus produce non-specific products. The results showed that these primer sequences were unique and should not have any significant non-specific binding. However, Fig. 1 shows two bands instead of one in the PCR products. This suggests that the primers actually did bind non-specifically to another part of the chromosome. Even though the primer sequences are unique, other regions of the chromosome might have similar sequences such that the primers are able to bind with lower affinity under less stringent annealing temperatures. Increasing the annealing temperature to 69.8 ºC significantly reduced the level of the non-specific product (Fig. 1, lane 7). This implies that the

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problem of non-specific binding of the primers can be eliminated by increasing the stringency.

The band in Fig. 1, lane 7 corresponds to the expected size of the *ompA* PCR product. Further confirmation that this PCR product is indeed the desired *ompA* gene involved digesting the PCR sample with *Hae*II, which cuts at two sites of the expected *ompA* PCR product. The expected results of the *Hae*II digestion of the PCR product are the presence of three bands, each with a size of 655 bp, 314 bp, and 190 bp. The gel showing the results of this digestion reveals the presence of these three expected bands, thus supporting that the product is the *ompA* gene (Fig. 2). The 190 bp band is faint due to the interference by the running dye. Lanes 2 and 3 are very similar except for the presence of extra faint bands in lane 2. This can be explained by the fact that the PCR products on which the restriction digest was performed for lane 2 (Fig. 1, lane 5) contained non-specific products which would have contributed to unexpected banding patterns. The PCR product in Fig. 1, lane 7 was then used to clone into pCR2.1 vector to create pCCK06-1. The pCR2.1 vector was chosen as the cloning vector because of its easy and efficient cloning method provided by the commercial Invitrogen TOPO TA Cloning kit. The presence of the inducible promoter P_{lac} and ampicillin resistance marker would allow a high-level of expression of *ompA* by IPTG induction and selection of transformants containing this plasmid.

The cloned fragment would insert in the multiple cloning site of the pCR2.1 vector, which is in the middle of *lacZα* gene (TOPO TA Cloning manual, Invitrogen, Carlsbad, CA). This insertion would inactivate *lacZα* and obliterate the *α*-complementation in the host cell that has Δ*lacZα* mutation (TOP10 competent cells) provided with the TOPO TA Cloning kit. Such insertional inactivation would result in the growth of white colonies in X-gal supplemented media, while absence of an insert would lead to blue colonies. This is because only a properly assembled LacZ (plasmid providing *lacZα* portion and host cell providing the *lacZω* portion) will cleave the X-gal substrate to produce the blue colour (5). Therefore, the TOP10 competent cells could be screened for the transformants containing plasmids that have an insert successfully ligated in them by blue-white screening (5). Unfortunately, the recipient strains *E. coli* 2320 and χ478 could not be subjected to blue-white screening because they have a complete deletion of *lacZ*, and cannot provide the *lacZω* portion. A substitute method for determining the presence of an insert in the transformed plasmid is to isolate the plasmid from the transformants, and restriction digest with *Eco*RI, and assess by agarose gel electrophoresis. *Eco*RI cleaves directly upstream and downstream of the cloned insert, and would therefore result in two DNA fragments: ~3.9 kb one containing most of the pCR2.1, and the other one slightly larger than the cloned insert.

Cloning results appear to be successful. After ligation of the PCR product into the pCR2.1, the resulting pCCK06-1 was transformed into TOP10 competent cells. Transformants were plated onto LB Amp media with X-gal and IPTG. Only the transformants were expected to grow because of the amp^R gene on the pCCK06-1 plasmid. The presence of X-gal and IPTG allowed the blue-white screening for the detection of the presence of a PCR insert in the plasmid. The results showed that the ratio of blue and white transformants was about 1:1. This suggests that the cloning efficiency was only about 50%, in contrast to the 95% efficiency claimed by the Invitrogen Cloning Kit (Invitrogen TOPO TA Cloning User Manual). This could be attributed to the fact that the *ompA* PCR product is fairly large, which reduces the efficiency of it positioning correctly within the nicked vector and ligating properly.

The plasmids in the white TOP10 transformant colonies are most likely pCCK06-1, which contain the *ompA* PCR insert. These plasmids were isolated from the white TOP10 transformant colonies, confirmed that it was indeed pCCK06-1 by restriction digest (Fig. 3), and electroporated into the two electrocompetent *E. coli* 2320 and χ478 recipient strains. This would introduce pCCK06-1 into the recipient cells. Transforming the recipient cells with the pCCK06-1 isolated from the white TOP10 transformant colonies instead of with the ligation mixture containing both pCCK06-1 and self-ligated pCR2.1 increases the efficiency of obtaining recipient transformants with pCCK06-1 in it. The subsequent isolation of plasmids from the transformed recipient cells and restriction digest with *Eco*RI showed that the transformed plasmids were indeed pCCK06-1 based on the two bands corresponding to the 3.9 kb pCR2.1 vector and the ~1.2 kb *ompA* PCR product (Fig. 4). These results show that construction of a plasmid with *ompA* gene cloned under inducible promoter and preparation of a conjugation recipient strain containing this plasmid were successful.

FUTURE EXPERIMENTS

The construct pCCK06-1 can be use to test the original purpose of how the increased level of OmpA in the recipient cells affects the conjugation efficiency. Two different recipient cells containing pCCK06-1 were prepared in this experiement. In the future, one can perform conjugation experiments using *E. coli* JCFLOxK1200 strain as the donor and the transformed *E. coli* 2320 or χ478 strains as the recipient. Prior to conjugation, the level of OmpA in the recipient cells should be checked to determine if it actually increased

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by isolation of membrane proteins followed by SDS-PAGE analysis and Western blotting using anti-OmpA antibodies, if available.

In order to compare the conjugation efficiency, the mating between donors and wild-type recipients must be done as the control, and the mating between donors and transformed recipients containing pCCK06-1 as the experimental conjugation. The introduction of pCCK06-1 is expected to increases the level of OmpA in the recipient cells, and hence affect the conjugation efficiency by changing the number of trasnconjugants from the control. Both conjugation mixtures can be plated onto LB Sm thi plates containing X-gal and IPTG to determine and compare the conjugation efficiencies.

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